A Draft Genome Sequence of *Pseudomonas syringae* pv. *tomato* T1 Reveals a Type III Effector Repertoire Significantly Divergent from That of *Pseudomonas syringae* pv. *tomato* DC3000

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Diverse gene products including phytotoxins, pathogen-associated molecular patterns, and type III secreted effectors influence interactions between *Pseudomonas syringae* strains and plants, with additional yet uncharacterized factors likely contributing as well. Of particular interest are those interactions governing pathogen-host specificity. Comparative genomics of closely related pathogens with different host specificity represents an excellent approach for identification of genes contributing to host-range determination. A draft genome sequence of *Pseudomonas syringae* pv. *tomato* T1, which is pathogenic on tomato but nonpathogenic on *Arabidopsis thaliana*, was obtained for this purpose and compared with the genome of the closely related *A. thaliana* and tomato model pathogen *P. syringae* pv. *tomato* DC3000. Although the overall genetic content of each of the two genomes appears to be highly similar, the repertoire of effectors was found to diverge significantly. Several *P. syringae* pv. *tomato* T1 effectors absent from strain DC3000 were confirmed to be translocated into plants, with the well-studied effector AvrRpt2 representing a likely candidate for host-range determination. However, the presence of *avrRpt2* was not found sufficient to explain *A. thaliana* resistance to *P. syringae* pv. *tomato* T1, suggesting that other effectors and possibly type III secretion system–independent factors also play a role in this interaction.

Additional keywords: effector-triggered immunity, nonhost resistance, pyrosequencing, Solexa sequencing.

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Many closely related plant pathogens, though adapted to fundamentally similar plant-associated niches, exhibit striking differences in the species and cultivars that they can infect. Host ranges of plant pathogens are governed by a complex network of interactions between plant defense mechanisms and pathogen gene products, with current models of plant defense emphasizing the roles of pathogen (or microbe) associated molecular pattern (PAMP)–triggered immunity (PTI) and effector-triggered immunity (ETI) (Jones and Dangl 2006).

Also referred to as basal resistance, PTI bears significant resemblance to the basal immunity exhibited by animals against various pathogens (Angot et al. 2007; Bhavsar et al. 2007; Zipfel 2008). PTI is typically triggered when the plant in question detects conserved elements of the microbial structure, such as flagellin (Felix et al. 1999), elongation factor Tu (Kunze et al. 2004), or lipopolysaccharides (Newman et al. 2002). Detection gives rise to a variety of defense-related phenotypes, including callose formation and upregulation of defense-related proteins, ultimately resulting in host-range restriction for a wide variety of microbes (Ham et al. 2007).

In contrast to the broad spectrum immunity conferred by recognition of the highly conserved PAMPs, ETI, also known as "gene-for-gene" resistance, involves detection of the more variable type III–secreted effector (T3E) proteins by plant resistance (*R*) gene products (Chisholm et al. 2006). Detection of effectors can occur directly or indirectly via the plant's detection of alterations brought about by effector actions and frequently results in the induction of programmed cell death (PCD) (Dangl and Jones 2001). The fact that individual effectors are generally more dispensable than PAMPs coupled with heavy selective pressure on the pathogen and host to alternately evade and achieve detection can lead to extensive variation in the repertoires of effectors and *R* genes among otherwise similar pathogens and hosts (Rohmer et al. 2004; Rose et al. 2007). Though ETI has been generally regarded as the basis for "host resistance" exhibited by individual plant cultivars and ecotypes to pathogen pathovars and

races, evidence is accumulating that ETI can also play a critical role in "nonhost resistance" exhibited by all cultivars and ecotypes of a plant species against all strains of a pathogen. For example, HopQ1-1 was recently shown to be the determining factor in interfering with pathogenicity of *P. syringae* pv. *tomato* DC3000 on *Nicotiana benthamiana* (Wei et al. 2007), and recognition of the effectors AvrPto1 (AvrPto) and HopAB2 (AvrPtoB) by the corresponding *R* genes appears to exclude tomato from the host range of several *P. syringae* strains (Lin and Martin 2007).

For many nonhost plant-pathogen interactions, the ultimate outcome is shaped by the interplay of multiple defense pathways and gene products. For example, resistance of *Arabidopsis thaliana* to the fungal wheat pathogen *Blumeria graminis* appears to involve PTI and ETI. During a prehaustorial phase, formation of papillae and callose layers typical of PTI is observed. During the posthaustorial phase, an induction of the hypersensitive response (HR) and consequent PCD typical of ETI is observed (Lipka et al. 2005).

Among bacterial pathogens, the outcomes of nonhost plant interactions may similarly involve diverse pathways, with T3E proteins involved at many levels. In fact, in addition to limiting host range through ETI, effectors also have the capacity to extend host range by suppressing defense pathways, including those implicated in PTI and ETI. In DC3000, several effectors as well as the toxin coronatine confer the ability to suppress induction of the PAMP-induced *A. thaliana NHO1* gene implicated in nonhost resistance (Li et al. 2005). *P. syringae* pv. *phaseolicola* 3121, which lacks coronatine and most of these effectors, is unable to suppress *NHO1* expression, likely contributing to its inability to cause disease on *A. thaliana*. Further evidence for the complexity of *A. thaliana* resistance to strain 3121 was uncovered by Ham and colleagues (2007), who found that strain 3121 elicits several parallel defense pathways in *A. thaliana*, with one of them being suppressed by the DC3000 effector HopM1.

To date, the genomes of three *Pseudomonas syringae* strains have been sequenced (Buell et al. 2003; Feil et al. 2005; Joardar et al. 2005) with bioinformatic analyses leading to the identification of over 40 T3E (Lindeberg et al. 2008). Of these strains, the tomato isolate DC3000 has been most extensively studied as a model pathogen of *A. thaliana* and tomato. Multilocus sequence typing (MLST) (Maiden et al. 1998) has revealed that DC3000 belongs to a group of *P. syringae* isolates found on members of the *Solanaceae* and *Brassicaceae* families that is characterized by the ability to cause disease on both *A. thaliana* and tomato (Yan et al. 2008). Other isolates, though closely related to DC3000, have been found exclusively on tomato and cause disease only on tomato but not on *A. thaliana* (Yan et al. 2008). The isolate T1, whose inability to cause disease on *A. thaliana* was first described by Whalen and colleagues (Whalen et al. 1991), is a representative of this latter group of typical *P. syringae* pv. *tomato* strains.

To investigate the genetic basis of T1's inability to cause disease on *A. thaliana*, a deep-coverage draft genome sequence of T1 was obtained using two high-throughput DNA sequencing technologies, pyrosequencing (Margulies et al. 2005), and Solexa sequencing (Bentley 2006). The draft genome sequence was annotated and analyzed for the presence of conserved and potentially novel T3E, for the purpose of identifying genetic differences that account for the differing abilities of DC3000 and T1 to infect *A. thaliana*. Although the two genomes were found to align for most of their length, their effector repertoires were found to be highly divergent. As a first step in dissecting *A. thaliana* resistance response to T1, the role of ETI in this response was investigated.

RESULTS

T1 is a nonpathogen of *A. thaliana* ecotypes and causes a HR.

Previously reported comparisons of disease symptoms and bacterial growth between *A. thaliana* and tomato strain DC3000, the typical *P. syringae* pv. *tomato* isolate T1, and other *P. syringae* isolates closely related to T1 revealed significant differences in host range. Specifically, the T1 population size on the *A. thaliana* ecotype 'Columbia' was shown to be approximately 4 log lower than that of DC3000 at 3 days post-infection and, in contrast to DC3000, T1 did not cause any disease symptoms (Yan et al. 2008). To evaluate whether this inability to multiply and to cause disease on *A. thaliana* is ecotype-specific or more widespread, comparisons of growth and symptom production were extended to three additional *A. thaliana* ecotypes and a type III secretion system (T3SS)-deficient derivative of DC3000 missing the *hrcC* gene (DC3000 CUCPB5112 [Penaloza-Vazquez et al. 2000]) was included as a control. As shown in Figure 1, T1 only grows to a population size equivalent to the T3SS-deficient DC3000 strain on all four *A. thaliana* ecotypes and does not cause any disease symptoms. Similar results were obtained with additional *P. syringae* pv. *tomato* isolates that are very closely related to T1, based on MLST (Yan et al. 2008), and additional *A. thaliana* ecotypes (data not shown). Therefore, *A. thaliana* resistance to T1-like isolates is not limited to a single ecotype-strain combination, suggesting that *A. thaliana* might be generally resistant to typical *P. syringae* pv. *tomato* strains.

While plant and bacterial factors contributing to *A. thaliana* resistance to T1 await characterization, infection of ecotype 'Columbia' with a high dose of T1 induces a HR, suggesting that ETI may contribute to the resistance exhibited by *A. thaliana* to the T1-like isolates (Fig. 1C). This is in agreement with the earlier observation (Whalen et al. 1991) that cosmids containing T1 genome fragments when expressed in DC3000 reduced virulence on *A. thaliana*.

T1 draft genome sequence obtained using Solexa sequencing and 454 pyrosequencing and compared with the DC3000 genome.

To initiate identification of the genetic bases for the differing virulence of T1 and DC3000 on *A. thaliana*, a draft genome sequence of *P. syringae* pv. *tomato* T1 was obtained using 454 pyrosequencing (Margulies et al. 2005) and Solexa sequencing (Bentley 2006). Reads were assembled separately into draft genome sequences. Characteristics of the two drafts are compared in Table 1. Based on the total contig length of the two draft genomes, the T1 genome was sequenced to a 20-fold coverage using 454 pyrosequencing and to a 94-fold coverage with Solexa sequencing. It is obvious that, although the Solexa genome coverage was more than four times greater than that of the 454 genome sequence, the longer pyrosequencing reads allowed a much more extensive assembly. To compare the quality of the two draft genome sequences, the two genomes were aligned and their average DNA identity was determined. The two drafts were found to be over 99.998% identical (1 difference every 100,000 bp) and are, thus, both of excellent quality. Moreover, all Solexa genome sequences were contained in the 454 genome sequence. Therefore, most of the subsequent analyses were conducted using the 454 draft genome sequence.

Given that the T1 454 draft genome sequence consists of 134 contigs, there are at least 134 gaps in the genome sequence. Most contig ends consist of repeated sequences, which is the reason why further assembly was not possible with current data. However, given the estimated genome coverage, we be-

lieve that the vast majority of the critical virulence genes, including effectors, are identifiable from the current draft.

Alignment of the T1 genome
with the three sequenced *P. syringae* genomes

To investigate differences between T1 and the other sequenced *P. syringae* strains, the T1 draft genome was aligned

Fig. 1. Comparison of in planta growth and symptoms of the *Arabidopsis thaliana* and tomato pathogen *Pseudomonas syringae* pv. *tomato* DC3000, a type III secretion system–deficient mutant of DC3000 (DC3000 T3SS–), and the tomato pathogen *P. syringae* pv. *tomato* T1. **A,** Bacteria were sprayed onto *A. thaliana* ecotypes Columbia (Col-0), Mt-0, Po-1, and WS at an optical density at 600 nm (OD$_{600}$) of 0.1. At 3 days postinfection, CFU per square centimeter were determined. While population sizes of DC3000 were significantly larger than those of DC3000 T3SS– and T1 on all ecotypes ($P < 0.0001$), sizes of DC3000 T3SS– and T1 were not significantly different from each other on any of the ecotypes, based on Student's *t*-test ($P > 0.1$). **B,** *A. thaliana* Col-0 plants were photographed a week postinfection. **C,** Bacteria were infiltrated into leaves of *A. thaliana* Col-0 at an OD$_{600}$ of 0.3, and pictures were taken after 16 h.
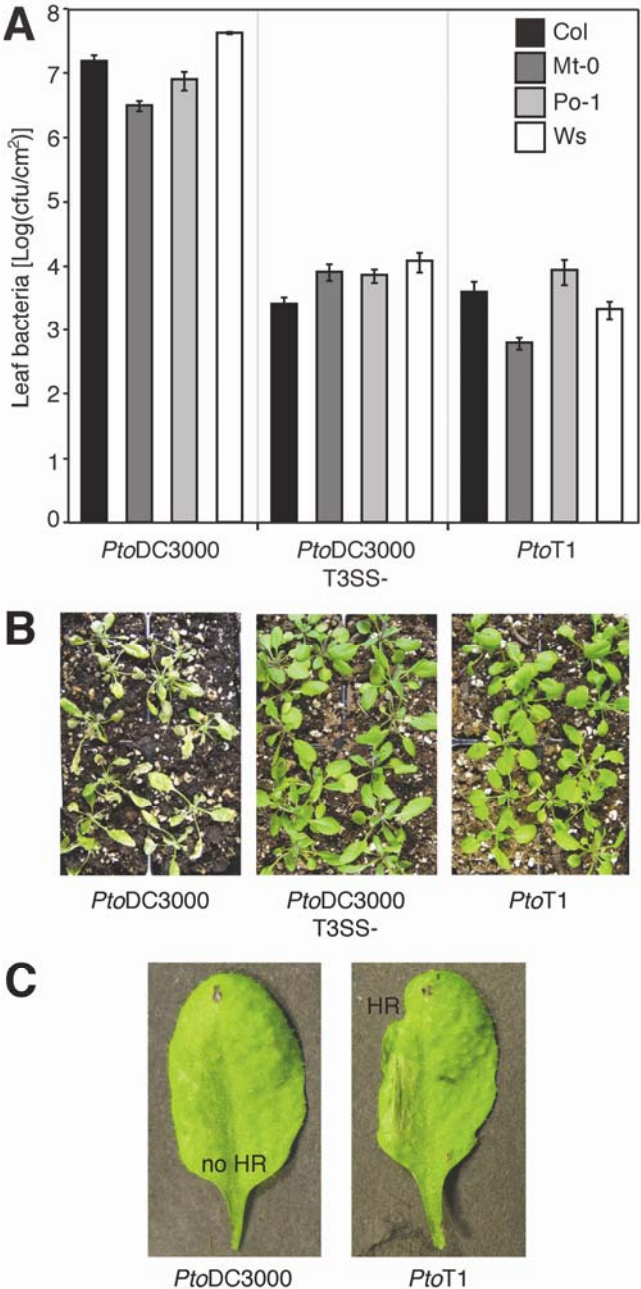
with the closed genomes of DC3000 (Buell et al. 2003), strain 1448A (Joardar et al. 2005), and *P. syringae* pv. *syringae* B728a (Feil et al. 2005).

The T1 genome was first compared with the DC3000 genome using the latter as reference. Figure 2 shows alignments of the DC3000 chromosome and its two plasmids pDC3000A and pDC3000B (panels A, B, and C, respectively) with both the 454 and the Solexa T1 draft genome sequences. It is evident from these figures that the DC3000 and the T1 genomes align over most of their length. Moreover, the alignments with the 454 and the Solexa T1 drafts are nearly identical, confirming the high quality of both genome sequences. As expected, alignment of the T1 draft genome sequence with the completely assembled B728a and 1448A genome sequences revealed significant divergence relative to the alignment of the two pathovar tomato strains. While 5,618,731 nt of DNA align between T1 and DC3000, only 4,547,158 nt of T1 align with the B728a genome and 4,689,063 nt align with the 1448A genome.

Comparison of protein complements
between the four *P. syringae* genomes.

In order to identify the repertoire of putative virulence genes in the T1 genome and to reveal differences between the DC3000 and T1 genomes that underlie differences in host range, an annotation of the T1 draft genome was generated. The annotation can be accessed at the DDBJ/EMBL/GenBank database as part of WGS project ABSM00000000 (version ABSM01000000). A total of 5,733 putative genes were predicted in this preliminary annotation, of which 3,987 (69%) are similar to genes that encode proteins with known function in other bacterial genomes, 729 (13%) are similar to genes that encode proteins with unknown function in other genomes ("conserved hypothetical proteins"), and 1,047 (18%) putative genes are unique to T1 ("hypothetical proteins"). However, it should be noted that estimates of the numbers of repeated elements such as mobile genetic elements are less reliable owing to the unclosed nature of the genome.

The predicted proteins from T1 were compared with those of the other sequenced *P. syringae* genomes. A Venn diagram illustrating the number of predicted proteins shared between and unique to T1 and DC3000 relative to the B728a and 1448A protein repertoires is shown in Figure 3. In summary, 4,271 proteins are shared between all four genomes and thus represent conserved housekeeping gene products and virulence gene products most likely underlying fundamental adaptations to the plant-associated niche. A total of 471 proteins are only shared between DC3000 and T1 and are absent from B728a and 1448A and may include proteins with roles in pathogenicity on the shared host tomato. A total of 757 proteins are unique to T1. Of these, 511 are hypothetical proteins, 79 proteins can be classified as mobile genetic elements (transposons, insertion sequences, bacteriophage components, and other genes with predicted function in DNA replication), and 167 are proteins with other predicted functions. This latter category

Table 1. Comparison of the *Pseudomonas syringae* pv. *tomato* T1 draft genome sequences obtained by 454 pyrosequencing and Solexa sequencing

Sequence characteristics	454	Solexa
Number of reads	519,729	15,723,622
Mean length of reads (nt)	241	36
Total length of reads (nt)	125,281,22	566,050,392
Number of assembled contigs	134	3,825
Mean length of contigs (nt)	45,878	1,570
Length of longest contig (nt)	335,36	16,815
Total length of contigs (nt)	6,147,659	6,005,250
Calculated genome coverage	20.4	94
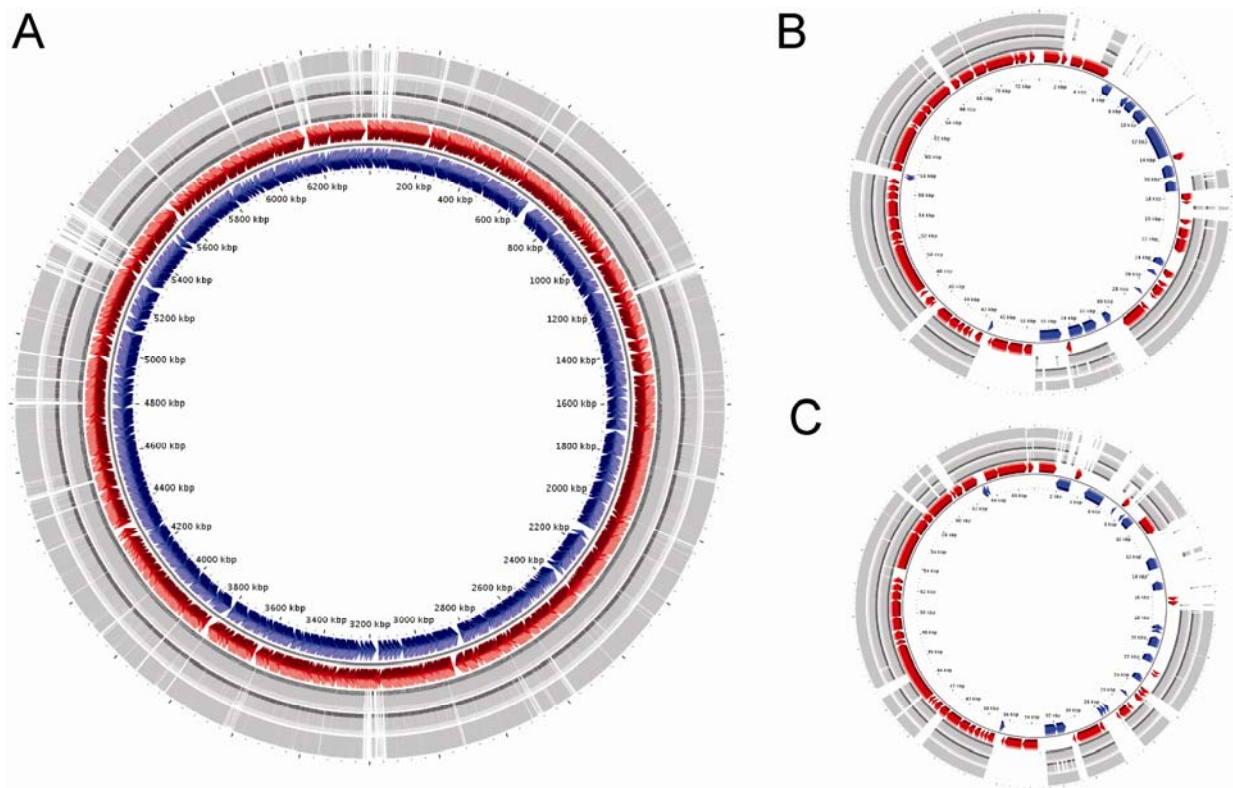
Fig. 2. Pairwise alignments between the *Pseudomonas syringae* pv. *tomato* T1 454 and Solexa draft genome sequences and the *P. syringae* pv. *tomato* DC3000 genome. **A,** Alignment of the T1 draft genome sequences with the DC3000 chromosome using the latter as reference. **B** and **C,** Alignment of the T1 draft genome sequences with the DC3000 plasmids pDC3000A and pDC3000B, respectively. Genes in the reference genome are in blue and red. Plotted Going outwards in each panel as follows: alignment with T1 Solexa contigs, alignment with T1 454 contigs, and single nucleotide polymorphism based on the Solexa consensus sequence. Closeups of local alignments corresponding to the type III–secreted effectors can be viewed on the PtoT1 genome website.
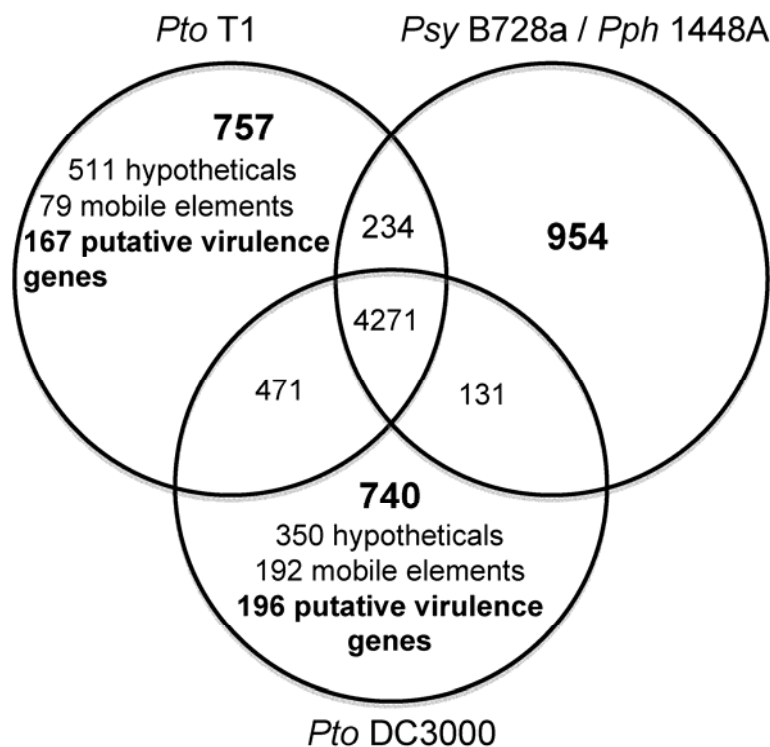
Fig. 3. Venn Diagram comparing the protein repertoire of *Pseudomonas syringae* pv. *tomato* (*Pto*) T1 with that of the completely sequenced genomes of *P. syringae* pv. *tomato* DC3000, *Pseudomonas syringae* pv. *syringae* (*Psy*) B728a, and *P. syringae* pv. *phaseolicola* (*Pph*) 1448A. Proteins from the B728a and 1448A genomes were combined. Numbers of predicted proteins for each section are indicated. An interactive version of this figure in which the different sections of the diagram are hyperlinked to corresponding lists of proteins are available on the Vinatzer lab's website.

represents the most interesting class of genes, with potential involvement in host specificity through either elicitation of *A. thaliana* defenses or contribution to virulence on tomato. DC3000 contains a similar number of gene products (196) that are unique among the four strains and have some functional annotation, which may account for the host range extension of DC3000 to *A. thaliana*. The hypothetical proteins unique to T1 or DC3000 may be found to also encode host specificity fac-

tors, if additional analyses are able to demonstrate which ones indeed code for expressed proteins.

A comparison of the predicted protein repertoire of T1 with all finished *Pseudomonas* genomes was also performed. Results can be queried in the supplemental data section at Virginia Tech's PtoT1 genome website.

Prediction of the T3SS effector repertoire of T1 and comparison with the DC3000 effector repertoire.

Comparison of major virulence factors identified in DC3000 (Lindeberg et al. 2008) with those encoded by T1 reveals that all major classes of virulence proteins appear to be present, with the exception of those encoding the toxin coronatine and a subset of T3E. To investigate the hypothesis that T3E are primarily responsible for the differences in host range observed between T1 and DC3000, a thorough analysis of T3E in T1 was conducted. The list of all known *P. syringae* effectors maintained at the Pseudomonas-Plant Interaction website was compared against the T1 genome using BLAST analysis. A subset of the T1 effector genes lacking orthologs in DC3000 (*avrA*, *avrRpt2*, *hopAS1*, and *hopAE1*) were cloned and expressed as Cya fusion proteins in DC3000, and translocation was confirmed as previously described (Schechter et al. 2004).

Table 2. In planta adenylate cyclase activity in *Nicotiana benthamiana* of *Pseudomonas syringae* pv. *tomato* T1 effector candidate-Cya fusion proteins

	pmol cAMP/μg protein	
Cya fusion protein	**DC3000 (T3SS+)[a]**	**5112 (T3SS−)**
AvrRpt2 (PSPTOT1_2469)	371.3 ± 47.8	19.1 ± 6.7
AvrA (PSPTOT1_4933)	191.3 ± 70.8	0.6 ± 0.2
HopAE (PSPTOT1_0516)	217.2 ± 17.8	0.7 ± 0.2
HopAS1 (PSPTOT1_1672)[b]	136.3 ± 11.6	0.6 ± 0.2
PSPTOT1_0854	15.3 ± 5.4	16.1 ± 5.2
EELORF2 (PSPTOT1_4759)	11.3 ± 3.2	10.7 ± 4.0

[a] T3SS = type III secretion system.
[b] Only the first 1 kb of coding sequence was tested.

Table 3. Repertoires of type III secretion system (T3SS) effectors and T3SS-independent genes in *Pseudomonas syringae* pv. *tomato* T1 compared with *P. syringae* pv. *tomato* DC3000[a]

DC3000	Locus tag	hrp Box	T1	Locus tag	hrp box
T3E orthologs deployed by both DC3000 and T1					
avrE1	PSPTO_1377	Yes	avrE1	PSPTOT1_0672	Yes
avrPtoB (hopAB2)	PSPTO_3087	Yes	hopAB3	PSPTOT1_1462	Yes
hopC1	PSPTO_0589	Yes	hopC1-1	PSPTOT1_1097	Yes
hopD1	PSPTO_0876	Yes	hopD1	PSPTOT1_2827	Yes
hopF2	PSPTO_0502	Yes (operon)	hopF2	PSPTOT1_0639	Yes (operon)
hopH1	PSPTO_0588	Yes	hopH1	PSPTOT1_1413	Yes
hopI1	PSPTO_4776	Yes	hopI1	PSPTOT1_0569	Yes
hopO1-1	PSPTO_A0018	Yes	hopO1-1	PSPTOT1_4763	Yes (operon)
hopQ1-1	PSPTO_0877	Yes	hopQ1-1	PSPTOT1_2826	Yes
hopR1	PSPTO_0883	Yes	hopR1	PSPTOT1_5535	Yes
hopT1-1	PSPTO_A0019	Yes (operon)	hopT1-1	PSPTOT1_4762	Yes (operon)
hopY1	PSPTO_0061	Yes	hopY1	PSPTOT1_1266	Yes
hopAA1-1	PSPTO_1372	Yes	hopAA1	PSPTOT1_0677	Yes
hopAF1	PSPTO_1568	Yes	hopAF1	PSPTOT1_4398	Yes
T3E orthologs deployed by DC3000 alone					
hopA1	PSPTO_5354	Yes (operon)	hopA1′	PSPTOT1_2530	Yes (operon)
hopB1	PSPTO_1406	Yes (operon)	hopB1′	PSPTOT1_0643	Yes (operon)
hopM1	PSPTO_1375	Yes (operon)	hopM1′	PSPTOT1_0674	Yes (operon)
avrPto	PSPTO_4001	Yes	Absent		
hopE1	PSPTO_4331	Yes	Absent		
hopG1	PSPTO_4727	Yes	Absent		
hopK1	PSPTO_0044	Yes	Absent		
hopN1	PSPTO_1370	Yes	Absent		
hopU1	PSPTO_0501	Yes	Absent		
hopV1	PSPTO_4720	Yes	Absent		
hopX1	PSPTO_A0012	Yes	Absent		
hopAA1-2	PSPTO_4718	Yes	Absent		
hopAM1-1	PSPTO_1022	Yes	Absent		
hopAM1-2	PSPTO_A0005	Yes	Absent		
hopAO1	PSPTO_4722	Yes	Absent		
T3E orthologs deployed by T1 alone					
hopS1′	PSPTO_4597	Yes (operon)	hopS1	PSPTOT1_0510	Yes (operon)
hopPtoAG:: ISPssy	PSPTO_0901	Yes	hopAG1	PSPTOT1_5517	Yes
hopAH1	PSPTO_0905	No	hopAH1	PSPTOT1_5516	Yes (operon)
hopAI1	PSPTO_0906	No	hopAI1	PSPTOT1_5515	Yes (operon)
hopAS1′	PSPTO_0474	Yes	hopAS1	PSPTOT1_1672	Yes
Absent			hopAE1	PSPTOT1_0516	Yes

[a] Reasons for lack of deployment include: absence of the structural gene; truncation, indicated by a single quotation; truncation by an insertion sequence, indicated by a double colon and the insertion sequence name; absence of a functional hrp promoter. Light gray background indicates that either one or both expression and translocation have not been fully evaluated; dark gray background indicates that either one or both expression and translocation are absent or unlikely; and no background shading indicates orthologs are likely functional.

The putative T1 effector genes *hopW1* and *avrD* were not tested for translocation, since these two genes are probably not contributing to *A. thaliana* resistance to T1; the effector gene *hopW1* is present in *P. syringae* pv. *maculicola* ES4326, well known to cause disease on many *A. thaliana* ecotypes, including Col-0, and *avrD* was shown not to reduce bacterial growth of ES4326 in *A. thaliana* (Wanner et al. 1993).

To identify those effector genes likely to be expressed and to identify any novel effectors that might be present in the T1 genome, a list of predicted binding sites for the HrpL alternate sigma factor was generated for the T1 genome as previously described (Fouts et al. 2002; Lindeberg et al. 2006; Vencato et al. 2006), and the candidate HrpL-regulated genes were analyzed for the presence of conserved N-terminal motifs consistent with type III secretion. Among the candidates with *hrp* promoters and plausible translocation motifs were T1 orthologs of the D3000 exchangeable effector locus open reading frame (ORF)2 gene product and of the *P. syringae* pv. *syringae* B728a gene encoding Psy_0737, though neither was found to be translocated. Cya test results are shown in Table 2. A list of the T1 *hrp* regulon and putative effector genes is shown in Table 3, together with the verified DC3000 *hrp* regulon and effector repertoire. T1 effectors have been added to the effector database at the Pseudomonas-Plant Interaction website.

Comparison of the effector repertoires for T1 and DC3000 reveals surprising differences despite the otherwise high degree of similarity and shared tomato host. Of the effectors that are likely to be deployed, as determined by the presence of an hrp box and intact coding region, only 14 are shared between the two, while as many as 15 are present only in the DC3000 genome and 11 only in the T1 genome. With regard to some of the best-studied T3SS effectors, T1 alone deploys AvrRpt2 (with only two amino-acid differences when compared with AvrRpt2$_{Pto\ JL1065}$, the allele originally cloned by R. Innes, [Innes et al. 1993]), while DC3000 alone produces AvrPto, HopU1, HopX1, and two HopAM1 paralogs. HopX1 is of particular interest, given that this gene is conserved in 1448a, B728a, and at least ten other *P. syringae* pathovars (Charity et al. 2003; Deng et al. 2003). HopM1 also appears to be deployed by DC300 alone, as the presence of a frameshift in the T1 ortholog reduces the likelihood of functionality. A homolog of HopAB2 (AvrPtoB) is encoded by both genomes but shows evidence of extensive divergence relative to flanking DNA, indicative of high selection pressure and of sufficient difference to warrant inclusion in a different subgroup (HopAB3) relative to the ortholog present in DC3000 (HopAB2).

Because of the draft status of the T1 genome sequence, there is uncertainty regarding a small number of effectors. The effec-

Table 3. *Continued from preceding page*

DC3000	Locus tag	hrp Box	T1	Locus tag	hrp box
T3E orthologs deployed by T1 alone (*continued*)					
Absent			hopW1	PSPTOT1_3130	Yes
Absent			avrA	PSPTOT1_4933	Yes
Absent			avrRpt2	PSPTOT1_2469	Yes
Absent			avrD1	PSPTOT1_0872	Yes
T3E orthologs deployed by neither DC3000 nor T1					
hopO1-3′	PSPTO_4592	No	hopO1-3	PSPTOT1_0506	No
hopT2	PSPTO_4590	No	hopT2	PSPTOT1_0505	No
hopAH2-1	PSPTO_3292	No	hopAH2-1	PSPTOT1_5346	No
hopAH2-2	PSPTO_3293	No	hopAH2-2	PSPTOT1_5345	No
hopD::IS52	PSPTO_4724	Yes	Absent		
hopQ1-2	PSPTO_4732	No	Absent		
hopAT1′	PSPTO_5618	No	Absent		
Absent			hopC1-2′	PSPTOT1_ 1415	Yes
Absent			hopAW (C-term)	PSPTOT1_4687	No
T3E status in DC3000 not fully evaluated					
hopO1-2	PSPTO_4594	Possible (operon)	hopO1-2	PSPTOT1_0508	Possible (operon)
hopS2	PSPTO_4588	Yes (operon)	hopS2	PSPTOT1_0503	Operon
hopT1-2	PSPTO_4593	Yes	hopT1-2	PSPTOT1_0507	Possibly (operon)
hopAN1	PSPTO_5061	No	hopAN1	PSPTOT1_3476	No
hopAD1	PSPTO_4691	Yes	Absent		
hopAQ1	PSPTO_4703	Possible	Absent		
Harpins, pilus-associated, helpers					
hrpA1	PSPTO_1381	Yes	hrpA1	PSPTOT1_0668	Yes
hrpK1	PSPTO_1405	Yes	hrpK1	PSPTOT1_0644	Yes
hrpW1	PSPTO_1373	Yes	hrpW1	PSPTOT1_0676	Yes
hrpZ1	PSPTO_1382	Yes	hrpZ1	PSPTOT1_0667	Yes
hopP1	PSPTO_2678	Yes	hopP1	PSPTOT1_5231	Yes
hopAK1	PSPTO_4101	Yes	hopAK1	PSPTOT1_2049	Yes
hrpH	PSPTO_1378	Yes	hrpH	PSPTOT1_0671	Yes
hopAJ1	PSPTO_0852	Yes	Absent		
Non-Hop hrp regulon (hrp promoters experimentally confirmed in other strains)					
apbE	PSPTO_2105	Yes	apbE	PSPTOT1_3672	Yes
Conserved hypothetical (truncated?)	PSPTO_5617	Yes	Conserved hypothetical	PSPTOT1_3153	Yes
MATE efflux	PSPTO_0370	Yes	MATE efflux	PSPTOT1_4882	Yes
iaaL	PSPTO_0371	Yes (operon)	iaaL	PSPTOT1_4881	Yes
Sugar transport	PSPTO_3489	Yes	Sugar transport	PSPTOT1_4786	Yes
Sugar transport	PSPTO_3488	Yes (operon)	Sugar transport	PSPTOT1_4787	Yes (operon)
Peptidase	PSPTO_0524	Yes	peptidase	PSPTOT1_1629	Yes
Conserved hypothetical	PSPTO_1409	Yes (operon)	Conserved hypothetical	PSPTOT1_0640	No
alc dehydrog operon	PSPTO_0834-36	Yes	Absent		
Hypothetical	PSPTO_4733	Yes	Absent		
Hypothetical	PSPTO_4733	Yes	Absent		

tor *hopD1* had a frameshift in the first 454 pyrosequencing assembly. However, upon resequencing the gene from a polymerase chain reaction (PCR) product, it became apparent that two full-length copies of the gene with one alternate nucleotide in position 927 were wrongly assembled into one contig and both nucleotides were integrated into the sequence. Only one copy of the gene is currently annotated, since we do not know the location of the second copy. Homologs of *hopC1* also appear to be present in two copies in T1; however, in this instance the two copies were assembled correctly in separate contigs. A frameshift error present in one of the two copies

was confirmed after resequencing the gene from a PCR product. The 412-bp-long 454 contig 00076 has similarity with the 3′ end of the *hopAW1*$_{Pph\ 1448A}$ gene. The 5′ end is not present in either the 454 or the Solexa draft genome sequences, suggesting that the copy of *hopAW1* in T1 is most likely a pseudogene.

The presence of *avrRpt2* in the T1 genome is not sufficient to explain the inability of T1 to cause disease in *A. thaliana*.

The observed HR caused by T1 on *A. thaliana* 'Columbia' and its inability to replicate and to cause disease on the other tested *A. thaliana* ecotypes suggests a "gene for gene" interaction between one of the T1-specific effectors and an *A. thaliana* resistance gene. The presence of the *avrRpt2* effector gene in the T1 genome makes the well-known interaction between *avrRpt2* and the cognate *A. thaliana* resistance gene *RPS2* (Mindrinos et al. 1994) the prime suspect. To evaluate the role of AvrRpt2$_{Pto\ T1}$ in resistance, DC3000, DC3000 expressing AvrRpt2$_{Pto\ JL1065}$, and T1 were compared for growth and presence of disease symptoms between *A. thaliana* 'Columbia and the *rps2* mutant line *rps2-101c* of *A. thaliana* 'Columbia (Mindrinos et al. 1994). As shown in Figure 4A, T1 population densities remained at a low level similar to those of the T3SSS-deficient DC3000 mutant (Fig. 1), even in the absence of the *avrRpt2-RPS2* interaction. However, no HR could be detected on *A. thaliana* 'Columbia' *rps2-101c* (Fig. 4B). These observations suggest that the *avrRpt2-RPS2* interaction is the source of the HR elicited by T1 on *A. thaliana* 'Columbia' but does not fully explain resistance of *A. thaliana* to T1.

Can gene for gene interactions other than *avrRpt2-RPS2* explain the inability of T1 to cause disease in *A. thaliana*?

Since gene for gene interactions are not always accompanied by a HR (Gassmann 2005), the absence of a HR during T1 infection of the *A. thaliana* 'Columbia' *rps2* mutant line does not exclude the possibility that ETI induced by T1 effec-
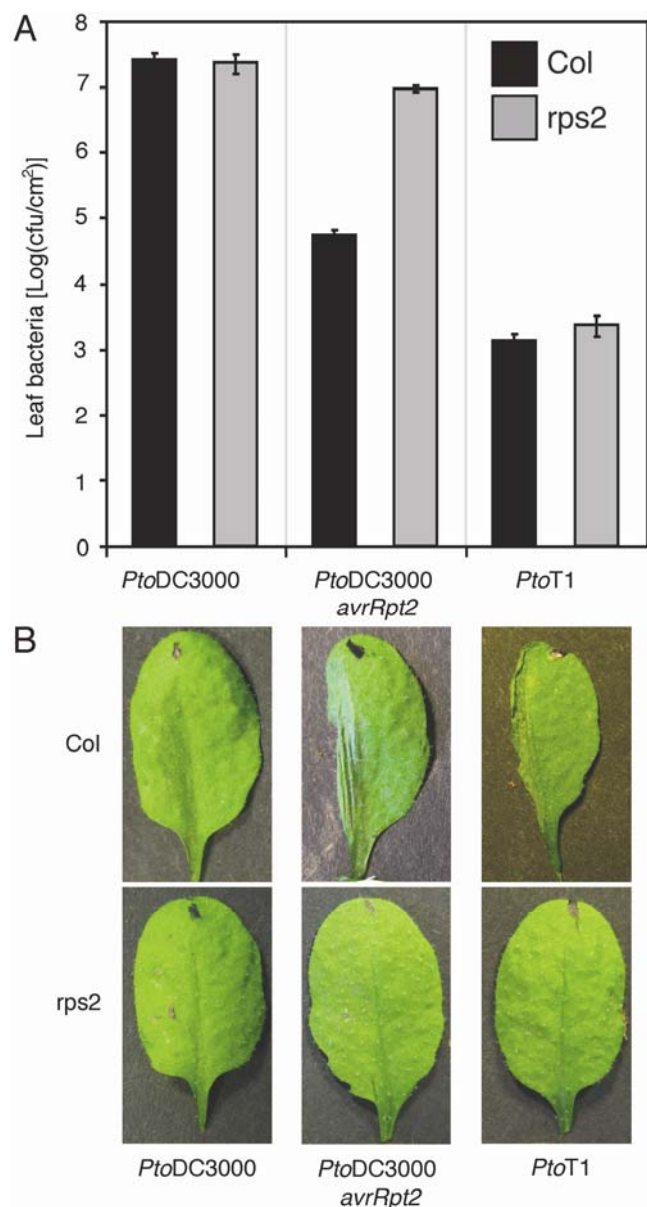
Fig. 4. Comparison of in planta growth of *Pseudomonas syringae* pv. *tomato* (*Pto*) DC3000, DC3000 expressing AvrRpt2, and T1 on *Arabidopsis thaliana* ecotype Col-0 and Col-0 *rps2-101c* mutated in the *RPS2* resistance gene. **A,** Plants were inoculated by spraying bacteria at an optical density at 600 nm (OD$_{600}$) of 0.1, and CFU per square centimeter was determined 3 days later for six leaf disks per isolate and plant. While DC3000 expressing *avrRpt2* grew statistically significantly more in the Col-0 *rps2-101c* plants than in the Col-0 plants ($P < 0.01$), growth of T1 was not statistically significantly different on the Col-0 *rps2-101c* plants comapred with Col-0 plants, based on Student's *t*-test ($P > 0.1$). **B,** Bacteria were infiltrated into leaves of *A. thaliana* Col-0 and Col-0 *rps2-101c* at an OD$_{600}$ of 0.3, and pictures were taken 16 h later.
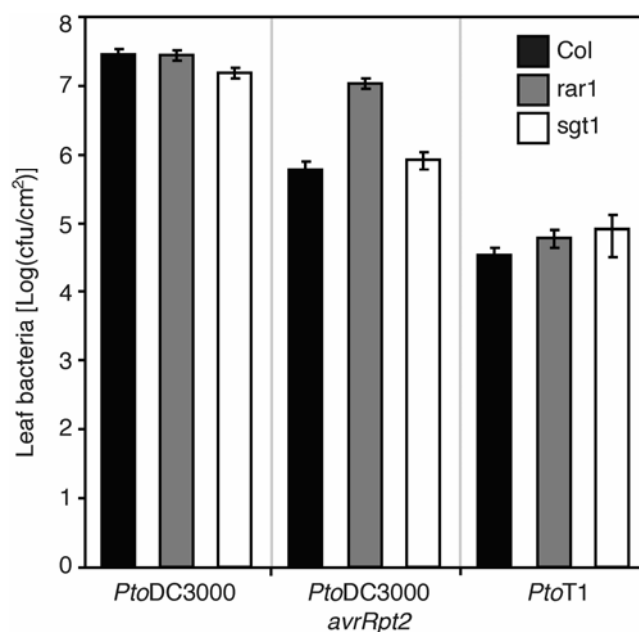
Fig. 5. Comparison of in planta growth of *Pseudomonas syringae* pv. *tomato* (*Pto*) DC3000, DC3000 expressing AvrRpt2, and *Pseudomonas syringae* pv. *tomato* T1 on wild-type *Arabidopsis thaliana* ecotype Col-0 and *A. thaliana* Col-0 mutated in the genes *SGT1* and *RAR1*, respectively. Plants were inoculated, and CFU were counted 3 days postinfection. While DC3000 expressing *avrRpt2* grew statistically significantly more in Col-0 plants mutated in *RAR1* than in Col-0 plants ($P < 0.0001$), growth of T1 was not statistically significantly different in plants mutated in *RAR1* or *SCT1* as compared with Col-0 plants, based on Student's *t*-test ($P > 0.1$).

tors other than AvrRpt2 is the determining factor in the inability of T1 to cause disease in *A. thaliana*. This possibility can be evaluated using *A. thaliana* lines mutagenized in the genes *RAR1* or *SGT1b*, previously shown to be essential for ETI in *A. thaliana* (Azevedo et al. 2006). Specifically, *SGT1b* was shown to be necessary for various *Hyaloperonospora parasitica*–*A. thaliana* gene for gene interactions and a mutation in *RAR1* was shown to reduce *A. thaliana* resistance to the *P. syringae* effectors AvrRpt2, AvrRpm1, and AvrRps4 (Austin et al. 2002). The *SGT1b* homolog *SGT1a* was not required for resistance triggered by these *P. syringae* effectors (Azevedo et al. 2006), nor could it complement a mutation in *SGT1b* during *Hyaloperonospora parasitica*–*A. thaliana* gene-for-gene interactions (Austin et al. 2002), except when overexpressed (Azevedo et al. 2006). Therefore, we reasoned that if ETI is induced during the T1–*A. thaliana* interaction by effectors other than AvrRpt2, T1 growth on *SGT1b* or *RAR1* mutant lines of *A. thaliana* might be increased. However, as shown in Figure 5, while growth of DC3000 expressing *avrRpt2* was increased 10-fold in the *RAR1* mutant, T1 growth was not significantly different in either the *SGT1b* or the *RAR1* mutant as compared with wild-type *A. thaliana* 'Columbia'. This was also true for the *P. syringae* pv. *tomato* isolate Max13 (Yan et al. 2008), from which *avrRpt2* is naturally missing, and for another *P. syringae* pv. *tomato* isolate with a disruption of *avrRpt2* (data not shown). Both isolates are identical to T1 based on our published MLST analysis (Yan et al. 2008). Therefore, while immunity triggered by AvrRpt2 and possibly other effectors contributes to *A. thaliana* resistance to T1 and similar *P. syringae* pv. *tomato* isolates, other mechanisms independent of ETI can be expected to play critical roles also.

DISCUSSION

Although T1 and DC3000 are both considered to be members of *P. syringae* pv. *tomato*, we have previously shown that they belong to two closely related but clearly distinct phylogenetic groupings with distinct host ranges (Yan et al. 2008). While T1-like strains cause disease only on tomato and are found on tomato with bacterial speck disease all over the world, DC3000-like strains cause disease on *A. thaliana*, cauliflower, and tomato and have been isolated mainly from family *Brassicaceae* and wild *Solanaceae* plants. Here, we show that resistance of *A. thaliana* to T1-like strains occurs in several ecotypes and that T1-like strains only reach population densities similar to a T3SS-deficient mutant of DC3000. These findings suggest that T1-like strains share a fundamental difference relative to DC3000 that prevents them from being pathogens of *A. thaliana*.

To date, the basis of nonhost resistance exhibited by *A. thaliana* toward *P. syringae* strains has been best studied using strains of *P. syringae* pv. *phaseolicola* (de Torres et al. 2006; Ham et al. 2007; Lu et al. 2001). However, the large number of differences between the *P. syringae* pv. *phaseolicola* strains and DC3000 greatly complicates identification of pathogen-host interaction factors using genome comparison. In contrast, the T1 genome exhibits a high level of similarity to DC3000 with the majority of differences consisting of short hypothetical

genes distributed throughout otherwise colinear regions. This suggests that many of these hypothetical genes may not be true coding sequences.

A surprising outcome of this study is that, while the two *P. syringae* pv. *tomato* genomes exhibit a high level of overall similarity, their repertoires of T3SS effectors are strikingly diverse. This indicates that T3SS effectors may play an important role in determining the difference in host specificity between the two strains, particularly with regard to *A. thaliana*. Effectors present in T1 but absent in DC3000 could lead to gene for gene resistance of *A. thaliana* to T1, while effectors present in DC3000 but missing in T1 could account for the inability of T1 to suppress ETI or PTI. Although T1 also lacks biosynthetic genes for the phytotoxin coronatine, this toxin has no demonstrated role in host range determination.

Of the 11 effectors present in the T1 genome but not in DC3000, the most notable is the well-characterized AvrRpt2, indirectly recognized in *A. thaliana* by the resistance gene product RPS2 upon degradation of RIN4 (Axtell and Staskawicz 2003; Kim et al. 2005; Mackey et al. 2003). However, it has been shown here that the *avrRpt2-RPS2* gene for gene interaction is not the sole mechanism of *A. thaliana* resistance to T1, since T1 growth on *A. thaliana* mutated in the *RPS2* gene is not increased compared with *A. thaliana* wild-type plants and symptoms are not increased. Moreover, additional putative gene for gene interactions are also insufficient to explain resistance of *A. thaliana* to T1, since the genes *RAR1* and *SGT1*, known to contribute to gene for gene resistance, are not necessary for this resistance. Therefore, we hypothesize that *A. thaliana* resistance to T1 is based on multiple parallel defense pathways, as previously observed for the *P. syringae* pv. *phaseolicola*–*A. thaliana* interaction (Ham et al. 2007). To identify the pathways involved, future work will be directed towards expression of DC3000 effectors in T1 and evaluation of their phenotypes in various single- and multiple-defense mutants of *A. thaliana*.

The two DC3000 effectors that probably have the greatest potential of conferring increased virulence of T1 on *A. thaliana* are *avrPto1*$_{PtoDC3000}$ and *hopM1*$_{PtoDC3000}$, which have both been shown to be important virulence factors during DC3000 infection of *A. thaliana* (Hauck et al. 2003; Nomura et al. 2006). However, the *P. syringae* pv. *tomato* isolate JL1065, which is closely related to the T1-like strains (although phylogenetically distinct [Yan et al. 2008]) expresses AvrPto1 as well as HopM1 (data not shown) but is still unable to cause disease on *A. thaliana* even in the absence of the *avrRpt2-RPS2* gene for gene interactions (Yan et al. 2008). Moreover, *hopM1* and *avrE1* have redundant function in the DC3000–*A. thaliana* interaction (DebRoy et al. 2004) and *avrE1* is present in the T1 genome (DNA identity, 95%). This makes it unlikely that simply expressing hopM1$_{Pto DC3000}$ in T1 could render T1 pathogenic on *A. thaliana*. Nonetheless, we performed this experiment and, as expected, could not observe any increase in bacterial growth or formation of disease symptoms in *A. thaliana* 'Columbia' (data not shown).

Based on the results obtained so far, we predict that identification of gene products capable of conferring increased growth or symptomology of T1, or both, in *A. thaliana* will require the

Table 4. *Pseudomonas syringae* pv. *tomato* isolates used in this study

Isolate name	Description	Reference
DC3000	*Arabidopsis thaliana* and tomato pathogen; rifampicin-resistant derivative of strainNCPPB 1106, isolated from tomato on the island of Guernsey (U.K.) by R. A. Lelliott.	Buell et al. 2003; Cuppels 1986; Yan et al. 2008
DC3000 CUCPB5112	Type III secretion system–deficient DC3000, because of deletion of the *hrcC* gene	Penaloza-Vazquez et al. 2000
DC3000 pLH12	DC3000 expressing AvrRpt2$_{Pto JL1065}$ from its native promoter on plasmid pLAFR3	Whalen et al. 1991
T1	Isolated from tomato in Canada by G. Bonn (Agriculture & Agri-Food Canada)	Whalen et al. 1991; Yan et al. 2008

simultaneous mutation of several *A. thaliana* defense pathways in combination with expression of one or more DC3000 effectors in T1. It is also possible that the inability of T1 to grow and cause disease on *A. thaliana* is due to yet unknown incompatibility mechanisms that are independent of characterized defense pathways and effectors, for example, gene for gene resistance independent of *RAR1* and *SGT1b* or nutritional requirements of T1 not met by the *A. thaliana* apoplast. The identified gene differences between T1 and DC3000 and allelic differences between shared genes represent an ideal unbiased starting point for experimental characterization of these mechanisms.

In conclusion, this study demonstrates that the comparison of a high quality *P. syringae* draft genome sequence with a completely assembled genome sequence of a closely related strain represents a powerful approach for identification of differences in gene content between strains. In particular, draft genome sequencing is an effective and rapid means of identifying the complete effector repertoire of individual *P. syringae* strains when used in combination with BLAST analysis and experimental testing of effector candidates identified by proximity to hrp box motifs. Experimental dissection of the roles of strain-specific effectors and other candidate host specificity genes identified through genome comparison is expected to reveal important insights into the specific interactions that govern differences in host range observed for the two strains compared here. Additional draft genome sequences currently being generated for other *P. syringae* strains by our group and by other researchers can be expected to take *P. syringae* genomics to new levels, accelerating the discovery of yet unknown aspects of molecular *P. syringae*–plant interactions.

MATERIALS AND METHODS

Bacterial strains and growth media.

Bacterial strains are listed in Table 4. *P. syringae* isolates were grown at 28°C on King's broth (King et al. 1954) agar plates supplemented with the following antibiotics at the following concentrations when necessary to maintain plasmids: kanamycin (50 μg/ml), tetracycline (15 μg/ml), and gentamycin (10 μg/ml).

Plant growth and infections.

For bacterial growth in planta and disease assays, *A. thaliana* plants were grown with a 16-h day and temperature was kept at 23°C during the day and 19°C during the night. Plants were infected at an age of 3 weeks, and bacterial population sizes were determined 3 days after infection as previously described (Yan et al. 2008). Differences between population sizes were statistically analyzed in the JMP 7 program (SAS Institute, Cary, NC, U.S.A.) using Student's *t*-test.

Genome sequencing and assembly.

For 454 sequencing, T1 (G. Martin lab glycerol stock #3876) was grown overnight in 1 ml of liquid media. Genomic DNA was extracted using a Wizard DNA purification kit (product #A1120; Promega, Madison, WI, U.S.A.) and by following the manufacturer's protocol for isolation of genomic DNA from gram-negative bacteria. The optical densities at 260 and 280 nm were determined using a NanoDrop ND-1000 spectrophotometer and were found to be 2.08. A total of 60 μg of DNA was precipitated in ethanol and 3 M NaAC. An aliquot of 10 μg of DNA was then used for single-stranded template DNA library construction and a titration run was carried out on the GS FLX sequencer (Roche, Basel, Switzerland) in accordance with the manufacturer's protocol. It was determined that the ratio of one DNA molecule per bead was the optimum.

Subsequently, a bulk emulsion PCR was carried out using this ratio, to provide the beads to run a full LR 70 plate. A total of one 7-h run was performed. The quality filtered reads were then assembled into contigs using the Newbler assembler.

For Solexa sequencing, DNA was prepared from bacteria grown in L medium using the Puregene Genomic DNA purification kit (Gentra Systems, Inc., Minneapolis) according to manufacturer's instructions. Libraries for single-read Illumina sequencing were prepared from 5 μg of DNA using a Genomic DNA sample prep kit (Illumina, Inc., Cambridge). DNA was fragmented by nebulization for 6 min at a pressure of 32 psi. For end-repair and phosphorylation, sheared DNA was purified using the QIAquick nucleotide removal kit (Qiagen, Crawley, U.K.). The end-repaired DNA was A-tailed and adaptors were ligated according to manufacturer's instructions. Size fractionation and purification of ligation products was performed using a 5% polyacrylamide gel run in tris-borate-EDTA at 180 V for 120 min. Gel slices were cut containing DNA in the 130- to 150-bp range. DNA was than extracted using 0.3 M sodium acetate and 2 mM EDTA (pH 8.0), followed by ethanol precipitation. Using 18 PCR cycles with primer 1.1 and 2.1 (Illumina, Inc.), 5′ adaptor extension and enrichment of the library was performed. The library was finally purified using a QIAquick PCR purification kit and adjusted to a concentration of 10 nM in 0.1% Tween. Flow cells were prepared according to manufacturer's instructions using cluster generation kits and a cluster station. Sequencing reactions were performed on a 1G genome analyzer (Illumina, Inc.). A total of 5 pM of the library were used to achieve approximately 25,000 to 30,000 clusters per tile. Sequence reads were assembled using Velvet 0.5 (Zerbino and Birney 2008).

Annotation.

The T1 draft genome was annotated by first blasting all open reading frames longer than 33 amino acids against all sequenced genomes in genera *Pseudomonas*, *Ralstonia*, and *Xanthomonas* available at National Center for Biotechnology Information (NCBI) in September, 2007; and all T3SS effector genes from the *P. syringae* effector database at the Pseudomonas-Plant Interaction website, using the GRC program (A. Warren and J. C. Setubal, *unpublished*). All ORF with no hits were then blasted against the nonredundant database at NCBI and were analyzed using InterProScan (Quevillon et al. 2005).

Comparative genomics.

The T1 454 and Solexa draft genome sequences were aligned against the published sequence of DC3000 (Buell et al. 2003), using blastn from the NCBI BLAST software suite (Altschul et al. 1997) using default parameters except -e 1e-10 -b 1 -v 1. Circular genome views of the alignments were generated using CGView (Stothard and Wishart 2005).

Protein repertoires were compared using pairwise blast (Altschul et al. 1997), 3GC (Telles et al. 2005) (used for the Venn diagrams shown in Figure 3), and OrthoMCL (Li et al. 2003), the latter of which clusters proteins in orthologous groups based on the MCL algorithm (Enright et al. 2002).

Effector prediction, cloning, *P. syringae* transformation, and testing for translocation.

T3SS effectors and other genes under the control of the HrpL sigma factor were predicted as described by Vencato and associates (2006). Effectors were cloned using the Gateway (Invitrogen, Carlsbad, CA, U.S.A.) cloning system into pENTR/SD/D-TOPO (primers available upon request), were recombined into the *P. syringae* vectors pBBRPnpt-GW-Cya and expressed in DC3000, and were tested for translocation as previously described (Schechter et al. 2004).

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AUTHOR-RECOMMENDED INTERNET RESOURCES

PtoT1 genome website: genome.ppws.vt.edu/pseudomonas
Pseudomonas-Plant Interaction website: pseudomonas-syringae.org
Newbler assembler: www.454.com